

REMARKS

In view of the above amendments and the following remarks, the Examiner is respectfully requested to withdraw the rejections and allow Claims 16 to 24, the only claims pending and under examination at this time.

The specification has been amended to insert the phrase "United States Patent Nos." prior to the list of patent numbers at pages 6 and 13.

Claim 16 has been amended to further clarify the claim language. Attached hereto is a marked up version of the changes made to the claims by the current amendment. The attached page is captioned **"Version with Markings to Show Changes Made"**. As can be seen from the above remarks and the attached copy of the marked up claims, no new matter has been introduced to the application by the above amendments. As such, the Examiner is respectfully requested to enter the above amendments.

With respect to the issue concerning the specification (item 5) appearing on page 4 of the office action, this issue has been addressed through the above amendment.

With respect to the drawings, enclosed please find formal drawings.

Turning now to the rejections presented in the Office Action, Claims 16-24 were rejected under 35 U.S.C. § 112, 2nd ¶ for a number of reasons. Each of these issues, i.e., issues A, D and E, is addressed separately below:

A. The Examiner asserts that the phrase "of less than about" in Claim 16 renders the claim indefinite. In view of the removal of this phrase from the claim, this rejection may be withdrawn.

D. With respect to this issue, Claim 16 now more specifically recites that the claim is directed to treating a host by preventing a binding event between a first and second protein in a

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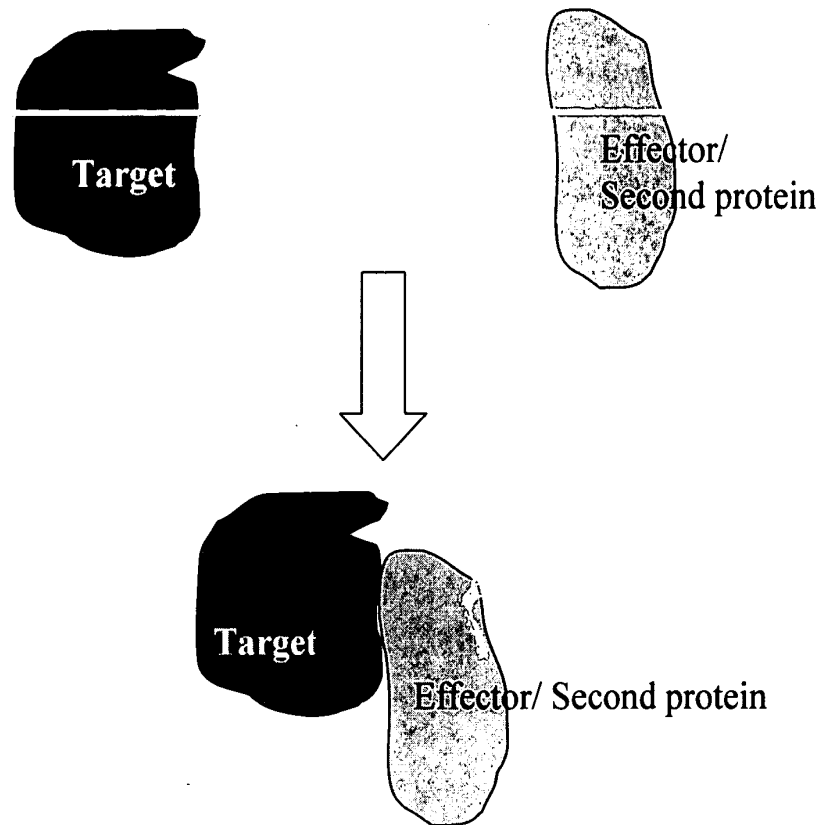
* host, i.e., in vivo, and that the production of the tripartite complex caused by administering an effective amount of the bifunctional molecule causes this binding event to be prevented, i.e., by blocking access of the second protein to the first protein. It is respectfully submitted that there are no missing steps, and therefore this issue has been addressed.

E. With respect to this issue, it is submitted that as amended, Claim 16 clearly recites that the formation of the tripartite complex prevents the binding of the first and second proteins from occurring. The specification further clarifies that the tripartite complex prevents access of the second protein to its binding site on the target protein through blocking of the binding site by the blocking protein of the tripartite complex. As such, this rejection may be withdrawn.

In view of the above remarks and amendments to the claims, it is believed that all of the issues upon which the Examiner rejected the claims under 35 U.S.C. § 112, 2nd ¶ have been addressed and this rejection may be withdrawn.

The Examiner has also rejected Claims 16 –24 under 35 U.S.C. § 112, 2nd ¶ for the asserted reason that the claimed methods lack essential steps. As pointed out above, the claims are clearly directed to methods of treating a host by inhibiting protein-protein interactions in a host for use in therapeutic applications. As such, the claims do not omit any essential steps and this rejection may be withdrawn.

Prior to addressing the remaining issues in the office action, it is believed that a brief review of the invention is helpful. The invention is directed to methods of inhibiting a protein-protein interaction in a host, e.g., for therapeutic purposes. In other words, the invention is directed to the field of inhibitor compounds for use in therapeutic applications. For example, the invention is directed to methods of inhibiting a biochemical event caused by two proteins, e.g., a target protein and an effector protein (which effector protein is referred to as a second binding protein in the above claims), by inhibiting the binding of the second protein to the target protein. The targeted binding event between a target protein and an effector/second binding protein that is the subject of the subject methods is illustrated below:



Traditionally, such methods have been accomplished using inhibitor molecules. However, the size of the inhibitor molecule needed to provide for the blocking activity can be limiting with respect to practical use in therapeutic applications. As such, there has been an ongoing need in the field to identify small molecule inhibitors.

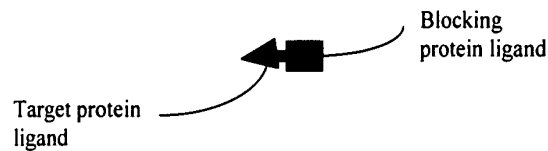
The present invention is based on the ingenious manner in which the inventors have satisfied this need for a small molecule effective inhibitor. In the present invention, a bifunctional molecule that recruits a blocking protein in vivo to produce an inhibiting complex is

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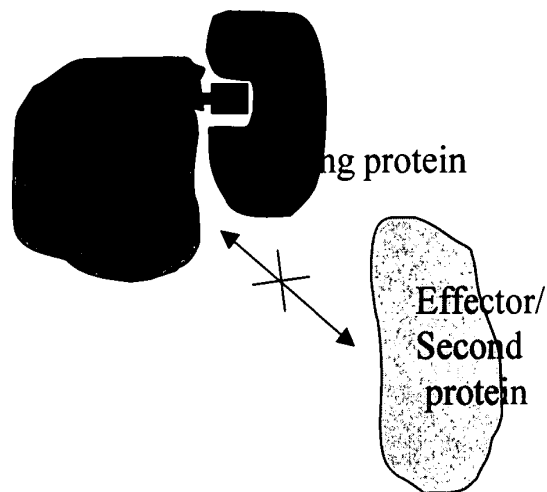
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employed. The bifunctional molecule is made up of target protein ligand and a second ligand that binds to a blocking protein. A representation of the bifunctional molecules employed in the subject invention is provided below:



When administered to the host, the bifunctional molecule binds to the target protein and a blocking protein, thereby inhibiting binding of the effector or second binding protein to the target. This process of simultaneously and non-covalently binding to the target protein and the blocking protein and thereby inhibiting binding of the effector/second binding protein to the target is illustrated below:



As the bifunctional ligand is a small molecule, i.e., less than 5000 daltons, that nonetheless turns into an effective inhibitor complex when it binds to the blocking protein, it satisfies the above need felt in the field of pharmaceutical inhibitor active agents.

Turning now to the remaining prior art rejections, the rejection of Claims 16 to 24 under

35 U.S.C. §102(b) as being anticipated by Griffith was maintained. In maintaining this rejection, the Examiner equates the described FK506-FKBP12 complex of Griffith with the bifunctional molecule employed in the subject claims.

As such, the Examiner's position relies on the assumption that the FK506-FKBP12 complex is a single molecule, in which the FK506 and the FKBP12 are covalently bound to each other, and that this assumed single molecule meets all of the limitation of the bifunctional molecule as claimed in the pending claims.

~~However~~, the FK506-FKBP12 complex is not a single molecule, but instead a complex of two disparate molecules, i.e., FK506 and FKBP12, which are non-covalently bound to each other. At page 519 of the Griffith article, it is taught that the FK506-FKBP12 complexes were produced according to the method of Wilson et al., Acta Cryst. (1995) D51, 511-521. A copy of this publication is enclosed. As can be seen in the Wilson publication, the complex was produced by combining the purified recombinantly produced FKBP12 with the FK506 and allowed the two molecules to non-covalently bind to each other to produce the complex. See pages 513 to 515. This publication clearly demonstrates that the FK506-FKBP12 complexes are not single molecules, but instead two different molecules noncovalently and specifically specifically bound to each other. As such, the FK506-FKBP12 complexes are not bifunctional molecules as defined in the claimed methods, because the distinct components are not covalently bonded to one another.

(NOT IN CLAIMS)
taught in the spec pages 9 and 10.

Furthermore, FKBP12 is a protein having a molecular weight of at least 10,000 daltons. As such, any complex of FKBP12 and FK506 is going to have a molecular weight of more than 5,000 daltons. Accordingly, the FK506-FKBP12 complex also fails to meet the limitations of the bifunctional molecules claimed in the present methods because the weight of this complex far exceeds that of 5000 daltons.

Finally, the bifunctional molecule employed in subject claims is a non-naturally occurring bifunctional molecule. Since the cited FK506 and FKBP12 complex is a complex of two disparate and non covalently bound molecules, i.e., FK506 and FKBP12, both of which are

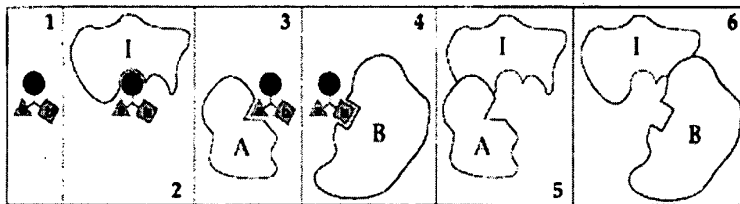
the combination of the two is not naturally occurring

naturally occurring (albeit recombinantly produced) and which naturally associate to produce the complex, the cited FK506-FKBP12 complex is a naturally occurring complex. Since the bifunctional molecule employed in the subject methods is a non-naturally occurring molecule, Griffith's FK506-FKBP12 complex fails to teach this aspect of the bifunctional molecules employed in the claimed methods as well.

The above discussion clearly demonstrates that Griffith fails to employ a bifunctional molecule as recited in the claimed methods. As such, Griffith does not anticipate that Claims 16-24 under 35 U.S.C. § 102(b) and this rejection may be withdrawn.

The rejection of Claims 16-21 and 24 under 35 U.S.C. §102 (b) as being anticipated by Varshavsky et al was also maintained. In maintaining this rejection, the Examiner argues that compound abi in figure 1 is a tripartite complex.






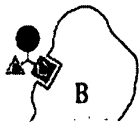


Figure 1 of Varshavsky is provided below:



The reference states that abi is made up of an "a" moiety; a "b" moiety and a "c" moiety all covalently bonded to each other to produce a trifunctional molecule. As such, abi is not a tripartite complex as produced in the claimed methods by a bifunctional molecule, a target protein and a blocking protein (i.e., three disparate molecules) since abi is a single molecule having three different moieties covalently bonded to each other ?

As such, contrary to the Examiner's reading, abi is not a tripartite complex as produced in the claimed methods, and the disclosure of abi does not anticipate the claimed methods.

The Examiner also asserts that Figure 2 discloses inhibiting a second protein with abi which anticipates the claimed methods and points to the interaction of abi and I. Figure 2 is provided below:

Cell type	Major complexes	Minor complexes	Toxicity of 
$A^+ B^+$			Low
$A^+ B^-$			Low
$A^- B^+$			Low
$A^- B^-$			High

However, as can be seen in Figure 2, when abi binds to I, only a binary complex made up of abi and I is produced. No tripartite complexes are produced in any of the schemes disclosed in Figure 2, because there are no complexes shown that are made up of three distinct moieties non-covalently but specifically bound to each other. *abi are distinct moieties.*

As such, and contrary to the Examiner's reading, abi bound to I as shown in Figure 2 is not a tripartite complex as produced in the claimed methods, and the disclosure of abi bound to I does not anticipate the claimed methods.

It is respectfully submitted that the Examiner is incorrectly reading the teachings of Varshavsky. Only binary complexes are produced in any of the schemes and figures shown or discussed in Varshavsky. None of the schemes discussed or shown in Varshavsky teach the

production of a tripartite complex produced by the non-covalent specific binding of a target protein, a bifunctional molecule and a blocking protein, much less one that prevents the binding of a target protein to a second binding/effector protein.

In fact, nowhere in the Varshavsky reference is the idea taught or suggested to make a tripartite complex of a target protein, a bifunctional molecule and blocking protein. Instead, all that is taught or suggested is binary complexes of either a bifunctional molecule and its target or a bifunctional molecule and a second protein that prevents the bifunctional molecule from binding to the target.

As such, Varshavsky fails to teach, or even suggest, a method in which a bifunctional molecule that produces a tripartite complex of a target protein and a blocking protein all non-covalently bonded to each other is produced. Accordingly, Varshavsky fails to anticipate Claims 16-21 and 24 under 35 U.S.C. §102 (b) and this rejection may be withdrawn.

Finally, Claims 22 and 23 have been rejected under 35 U.S.C. § 103(a) over Varshavsky in view of Pouletty, for the asserted reason that Varshavsky teaches all of the elements of the claimed method but for the extracellular production of tripartite complexes, which element is assertedly made up by Pouletty.

However, as pointed out above, Varshavsky is fundamentally deficient in failing to teach or even suggest the production of tripartite complexes. In fact, Varshavsky actually teaches away from the production of tripartite complexes for the following reasons. Varshavsky's whole paper is directed to ways of making drugs more selective. The approach suggested is to link the drug to a second ligand that will bind to a protein in a cell where one does not want drug activity, such that when the bifunctional molecule binds to the second protein in the cell where drug activity is not wanted, the bifunctional molecule cannot bind to the drug target. As such, drug activity is limited to those cells that lack the second protein. For this scheme to work, binding to the second protein, i.e., the blocking protein, must prevent the bifunctional molecule from binding to the target. If the bifunctional molecule binds to the target, one would still get drug activity and selectivity would not be achieved. **As such, Varshavsky's scheme only works if the**

bifunctional molecule cannot form a tripartite complex with a target protein and blocking protein at the same time, i.e., cannot simultaneously bind to a target protein and a blocking protein.

This requirement of Varshavsky is directly opposite to the claimed methods, where one must produce a tripartite complex between a target protein, a bifunctional molecule and a blocking protein.

As such, Varshavsky teaches away from the claimed methods because Varshavsky teaches methods in which tripartite complexes must not be produced, but instead only binary complexes are produced. **Because Varshavsky teaches away from methods in which tripartite complexes are produced, this reference fails to suggest the production of tripartite complexes, a required element of the claimed methods.**

As Pouletty has been cited solely for the extracellular production site, the Pouletty teaching is incapable of making up the above fundamental deficiency in Varshavsky.

In sum, because the combined teaching of Varshavsky and Pouletty fails to teach or suggest, and in fact teaches away from, a method in which a tripartite complex of a bifunctional molecule, a target protein and blocking protein is produced, Claims 22 and 23 are not obvious over the combined teachings of these references and this rejection of Claims 22 and 23 under 35 U.S.C. § 103(a) over Varshavsky in view of Pouletty may be withdrawn.

In view of the above amendments and remarks, this application is considered to be in good and proper form for allowance and the Examiner is respectfully requested to pass this application to issuance.

Atty Dkt. No.: STAN166
USSN: 09/716,054

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815.

Respectfully submitted,

Date: 8.5.02

By: 

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encs:

Formal Drawings
Wilson et al., 1995.

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Page 6, please replace the paragraph from page 6, line 30 to page 7, line 3, with the following :

As such, the target protein ligand may be obtained from a library of naturally occurring or synthetic molecules, including a library of compounds produced through combinatorial means, i.e. a compound diversity combinatorial library. When obtained from such libraries, the target protein ligand employed will have demonstrated an affinity for its protein target in an appropriate screening assay for the activity. Combinatorial libraries, as well as methods for the production and screening, are known in the art and described in United States Patent Nos: 5,741,713; 5,734,018; 5,731,423; 5,721,099; 5,708,153; 5,698,673; 5,688,997; 5,688,696; 5,684,711; 5,641,862; 5,639,603; 5,593,853; 5,574,656; 5,571,698; 5,565,324; 5,549,974; 5,545,568; 5,541,061; 5,525,735; 5,463,564; 5,440,016; 5,438,119; 5,223,409, the disclosures of which are herein incorporated by reference.

Page 13, please replace the paragraph from lines 26 to 33 with the following:

Alternatively, the bifunctional inhibitor molecule can be produced using combinatorial methods to produce large libraries of potential bifunctional molecules which may then be screened for identification of a bifunctional molecule with the desired binding affinity and/or specificity. Methods for producing and screening combinatorial libraries of molecules include United States Patent Nos: 5,741,713; 5,734,018; 5,731,423; 5,721,099; 5,708,153; 5,698,673; 5,688,997; 5,688,696; 5,684,711; 5,641,862; 5,639,603; 5,593,853; 5,574,656; 5,571,698; 5,565,324; 5,549,974; 5,545,568; 5,541,061; 5,525,735; 5,463,564; 5,440,016; 5,438,119; 5,223,409, the disclosures of which are herein incorporated by reference.

In the Claims:

16. (Twice Amended) A method ~~for~~ of treating a host by inhibiting a binding event between a first target protein and a second binding protein in a host, said method comprising:

administering to said host an effective amount of a non-naturally occurring bifunctional inhibitor molecule of less than ~~about~~ 5000 daltons consisting of:

(a) a target protein ligand that specifically binds to said first target protein; and

(b) a blocking protein ligand that specifically binds to a blocking protein,

wherein said target protein ligand and said blocking protein ligand are bonded to each other, optionally through a linking group; ~~wherein said bifunctional inhibitor molecule is capable of simultaneously binding said target protein and said blocking protein in a manner sufficient to inhibit said binding event;~~

to simultaneously bind said first target protein and said blocking protein to produce a tripartite complex comprising said bifunctional inhibitor molecule, said target protein and said blocking protein that inhibits said binding event of said second binding protein to said first target protein to treat said host.

IMMUNE SYSTEM APPLICATIONS OF STRUCTURE-AIDED DRUG DESIGN

Acta Cryst. (1995). D51, 511–521

Comparative X-ray Structures of the Major Binding Protein for the Immunosuppressant FK506 (Tacrolimus) in Unliganded Form and in Complex with FK506 and Rapamycin

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Abstract

FK506 (tacrolimus) is a natural product now approved in the US and Japan for organ transplantation. FK506, in complex with its 12 kDa cytosolic receptor (FKBP12), is a potent agonist of immunosuppression through the inhibition of the phosphatase activity of calcineurin. Rapamycin (sirolimus), which is itself an immunosuppressant by a different mechanism, competes with FK506 for binding to FKBP12 and thereby acts as an antagonist of calcineurin inhibition. We have solved the X-ray structure of unliganded FKBP12 and of FKBP12 in complex with FK506 and with rapamycin; these structures show localized differences in conformation and mobility in those regions of the protein that are known, by site-directed mutagenesis, to be involved in calcineurin inhibition. A comparison of 16 additional X-ray structures of FKBP12 in complex with FKBP12-binding ligands, where those structures were determined from different crystal forms with distinct packing arrangements, lends significance to the observed structural variability and suggests that it represents an intrinsic functional characteristic of the protein. Similar differences have been observed for FKBP12 before, but were considered artifacts of crystal-packing interactions. We suggest that immunosuppressive ligands express their differential effects in part by modulating the conformation of FKBP12, in agreement with mutagenesis experiments on the protein, and not simply through differences in the ligand structures themselves.

Introduction

FK506 (Prograf®; USAN tacrolimus) is a natural product screening lead (Kino *et al.*, 1987) that acts as a down-regulatory agonist on Ca²⁺-dependent activation pathways in T-cells, by interrupting transcription of interleukin-2 and other T-cell activation genes (Tocci *et al.*, 1989; Mattila *et al.*, 1990; Schreiber, 1991). This mechanism is dependent on the inhibition of calcineurin, an intracellular Ca²⁺-dependent phosphatase

(Klee & Cohen, 1988) by the FKBP12-FK506 complex (Liu *et al.*, 1991). FKBP12 also binds a related immunosuppressant, rapamycin (Vezina, Kudelski & Sehgal, 1975) (Rapamune®; AY22989; USAN tacrolimus), which inhibits lymphocyte-induced proliferation through a mechanism distinct from that of FK506 (Dumont, Staruch, Koprak, Melino & Sigal, 1990). The mechanism of action of immunosuppressive agents has been reviewed recently (Schreiber, 1991; Sigal & Dumont, 1992; Rosen & Schreiber, 1992; Schreiber & Crabtree, 1992; Schreiber, Albers & Brown, 1993; Liu, 1993a,b; Navia & Peattie, 1993a,b; Armistead & Harding, 1993).

Roughly half of the chemical structure of FK506 is found to be homologous in rapamycin, an observation that led to a renewal of interest in that ligand's immunosuppressive properties (Tocci *et al.*, 1989). FK506 and rapamycin can each antagonize the distinct immunosuppressive effects of the other by competing for FKBP12 through the common interaction of their 'binding domains'. The structure of the bound ligands has been characterized in detail by X-ray crystallography of the respective FKBP12–ligand complexes (Van Duyne, Standaert, Karplus, Schreiber & Clardy, 1991, 1993; Van Duyne, Standaert, Schreiber & Clardy, 1991; Rotonda, Burbaum, Chan, Marcy & Becker, 1993; Becker *et al.*, 1993). Those structural studies also showed that the non-homologous 'effector-domain' regions of the ligands protrude beyond the surface of the protein with distinct conformations that might well accommodate calcineurin binding and inhibition with FK506, but neither with rapamycin.

An elegant effector-domain model has been proposed (Schreiber, 1991; Rosen & Schreiber, 1992; Schreiber & Crabtree, 1992; Liu, 1993a,b; Schreiber *et al.*, 1993) to explain the different functional consequences of ligand binding to FKBP12 in terms of these structural features. [The FKBP12–rapamycin complex, common binding to FKBP12 notwithstanding, as well as free FK506, rapamycin, and the uncomplexed FKBP12 itself, are all ineffective as calcineurin inhibitors (Schreiber, 1991; Sigal & Dumont, 1992).] The model has enjoyed broad acceptance because it is consistent with the observed

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Table 1. Solved crystal structures

Resolution = resolution of the X-ray data. *R* factor = crystallographic residual. R.m.s. bonds and r.m.s. angles = root-mean-square deviation of bond lengths and angles from ideal values. The space group and unit-cell dimensions for each solved structure are given. R.m.s.d. = root-mean-square deviation for main-chain atoms after least-squares superposition with Nati-FK506 structure.

Crystals	Resolution (Å)	<i>R</i> factor (%)	R.m.s. bonds (Å)	R.m.s. angles (°)	Space group	<i>a</i> (Å)	<i>b</i> (Å)	<i>c</i> (Å)	α (°)	β (°)	γ (°)	R.m.s.d. main chain (Å)
Native	2.2	15.8	0.017	3.5	$P4_12_12$	34.1	34.1	202.2	90	90	90	0.51
Nati-FK506	1.7	10.4	0.014	4.7	$P2_12_12$	36.1	36.1	22.7	90	90	90	—
Nati-Rapa	1.7	18.1	0.016	2.9	$P2_12_12$	48.9	49.5	55.2	90	90	90	0.64
Complex 1	1.7	18.7	0.014	2.9	$R3$	41.9	41.9	41.9	83.9	83.9	83.9	0.63
Complex 2	2.3	17.5	0.019	3.7	$P2_1$	36.5	92.2	32.9	90	117.9	90	0.47
Complex 3	1.5	18.2	0.017	2.9	$P2_12_12$	34.9	40.6	80.6	90	90	90	0.46
Complex 4	1.9	18.5	0.017	3.1	$P2_12_12$	28.8	37.3	98.4	90	90	90	0.42
Complex 5	1.8	18.1	0.015	3.1	$P2_12_12$	29.7	52.4	85.1	90	90	90	0.53
Complex 6	1.9	19.2	0.017	3.2	$P2_12_12$	96.2	37.3	30.7	90	90	90	0.45
Complex 7	2.1	18.2	0.017	3.4	$P2_12_12$	32.5	45.3	67.9	90	90	90	0.39
Complex 8	1.8	18.9	0.014	3.1	$P2_12_12$	29.4	62.4	112.1	90	90	90	0.44
Complex 9	1.9	18.7	0.013	3.1	$P2_12_12$	30.6	59.6	66.7	90	90	90	0.41
Complex 10	2.1	20.1	0.015	3.3	$P2_12_12$	28.9	49.9	75.1	90	90	90	0.45
Complex 11	1.8	17.8	0.014	2.9	$P2_12_12$	94.9	36.4	33.5	90	90	90	0.43
Complex 12	2.2	18.7	0.015	3.3	$P2_12_12$	50.0	56.2	73.0	90	90	90	0.49
Complex 13	1.8	18.1	0.013	2.9	$P3_1$	56.2	56.2	79.8	90	90	120	0.49
Complex 14	1.8	18.9	0.013	3.1	$P2_12_12$	53.1	69.7	54.8	90	90	90	0.40
Complex 15	2.2	20.9	0.016	3.4	$P1$	45.0	42.9	37.3	90	101	108	0.47
Complex 16	1.8	20.5	0.012	1.8	$P2_12_12$	44.0	72.9	36.4	90	90	90	0.42

reciprocal antagonism of FK506 and rapamycin, because it can be extended to rationalize the reduction in calcineurin inhibitory activity that accompanies minor modifications of FK506 (Tocci *et al.*, 1989; Sigal & Dumont, 1992; Armistead & Harding, 1993; Organ *et al.*, 1993; Dumont *et al.*, 1992; Parson, Sigal & Wyvratt, 1993; Kawai *et al.*, 1993; Goulet, Rupprecht, Sinclair, Wyvratt & Parsons, 1994), and because it has had predictive value in ligand design. [Support for the model was provided, for example, by the synthesis of 506BD, a binding-domain mimic devoid of effector-domain structural elements, which proved to be an antagonist of FK506-mediated calcineurin inhibition (Bierer, Somers, Wandless, Burakoff & Schreiber, 1990; Somers, Wandless & Schreiber, 1991).]

In contrast to the pivotal role assigned to FK506 in the effector-domain model, the FKBP12 protein itself was left with the minimal function of a scaffold or 'presenter' (Schreiber, 1991) of ligands to calcineurin. This hypothesis was supported by a comparison of the structures of FKBP12 in its complexes with FK506 and with rapamycin, as determined by X-ray diffraction (Van Duyne, Standaert, Karplus *et al.*, 1991; Van Duyne, Standaert, Schreiber *et al.*, 1991; Van Duyne, Standaert, Karplus, Schreiber & Clardy, 1993), and of unliganded FKBP12, as determined by solution NMR methods (Michnick, Rosen, Wandless, Karplus & Schreiber, 1991; Moore, Peattie, Fitzgibbon & Thomson, 1991; Rosen, Michnick, Karplus & Schreiber, 1991), which suggested that the structure of the protein was unaffected in its conformation and mobility by ligand binding. Differences were observed between the three structures, but these were attributed to experimental artifacts, including crystal-packing interactions (Van Duyne, Standaert, Karplus

et al., 1991; Van Duyne, Standaert, Schreiber *et al.*, 1991; Van Duyne *et al.*, 1993) and a paucity of NMR constraints (Michnick *et al.*, 1991; Moore *et al.*, 1991; Rosen *et al.*, 1991), in specific regions of the protein, an assumption that would be prudent in a structural comparison involving a small protein like FKBP12 on the one hand, and a mix of structure-solution methodologies on the other.

Here, we report the structure of FKBP12 in its native unliganded state and in complex with FK506, with rapamycin, and with 16 other synthetic FKBP12-binding ligands (Table 1). A comparison of these structures shows that the conformation of FKBP12 can be locally modulated (Fig. 1), with differences comparable in magnitude to the structural differences observed in mutant FKBP12-FK506 complexes that are unable to inhibit calcineurin (Itoh *et al.*, 1995). These observations reinforce the view (Aldape *et al.*, 1992) that ligand-induced changes in the structure of FKBP12 may play a role in the calcineurin inhibitory potential of the protein-ligand complex.

Materials and methods

Bovine thymus FKBP12 (bFKBP12) purification

Fresh bovine thymus was trimmed and cut into approximately 2.5 cm³ cubes, frozen in liquid N₂ and stored at 203 K for later use. 3–4 kg of frozen tissue was thawed, 1 kg at a time, in 5 volumes of cold extraction buffer (100 mM potassium phosphate, 0.5 mM EDTA, 1 mM β -mercaptoethanol, 0.02% NaN₃, 0.1 mM PMSF) and homogenized using a Polytron (Brinkmann PT-6000, with large scale generator). After complete homogenization, chloroform was added to a final

concentration of 2.5%(v/v) and briefly mixed using the Polytron. The homogenate was centrifuged at 12 000g for 50 min at 278 K. The pelleted material, containing the majority of the excess chloroform, was discarded and the supernatants were combined and adjusted to 1.7 M potassium phosphate by addition of a 4.0 M (pH 7.1) stock. The solution was centrifuged again, as above, to remove any precipitated protein, and the entire supernatant from 3–4 kg of tissue was applied (now rate 15–20 ml min⁻¹, at 295 K) to a 5 × 30 cm hydrophobic interaction (HIC) column (Rainin Hydropore-HIC) which had been previously equilibrated in 1.7 M potassium phosphate, 1 mM β -mercaptoethanol, pH 7.1. After washing with two column volumes of the same buffer, the bound protein was eluted at 295 K with degassed dH₂O containing 1 mM β -mercaptoethanol. The eluted protein was concentrated by ultrafiltration to about 150 ml, and then fractionated by size-exclusion chromatography on a Pharmacia Sephacryl S-100 HR column (10 × 110 cm) equilibrated in extraction buffer.

The FKBP12-containing fractions (around 65–70% of the column volume) were pooled and then adjusted to 1.7 M potassium phosphate by addition of a 4.0 M (pH 7.1) stock. This material was loaded directly onto a second HIC column (Rainin Hydropore-HIC; 2 × 30 cm; flow rate 4 ml min⁻¹, at 295 K). The proteins were eluted at 4 ml min⁻¹ with a 3 h linear gradient into degassed dH₂O containing 1 mM β -mercaptoethanol. The FKBP12 peak, eluting around 1.1 M potassium phosphate, represented more than 90% of the protein. The final yield was typically about 10–12 mg per kg of tissue.

Human recombinant FKBP12 (hrFKBP12) expression and purification

High-level expression of hrFKBP12 in *E. coli* was achieved by construction of vectors containing the complete sequence for the gene, subcloned behind a phage T7 RNA polymerase promoter vector (Studier,

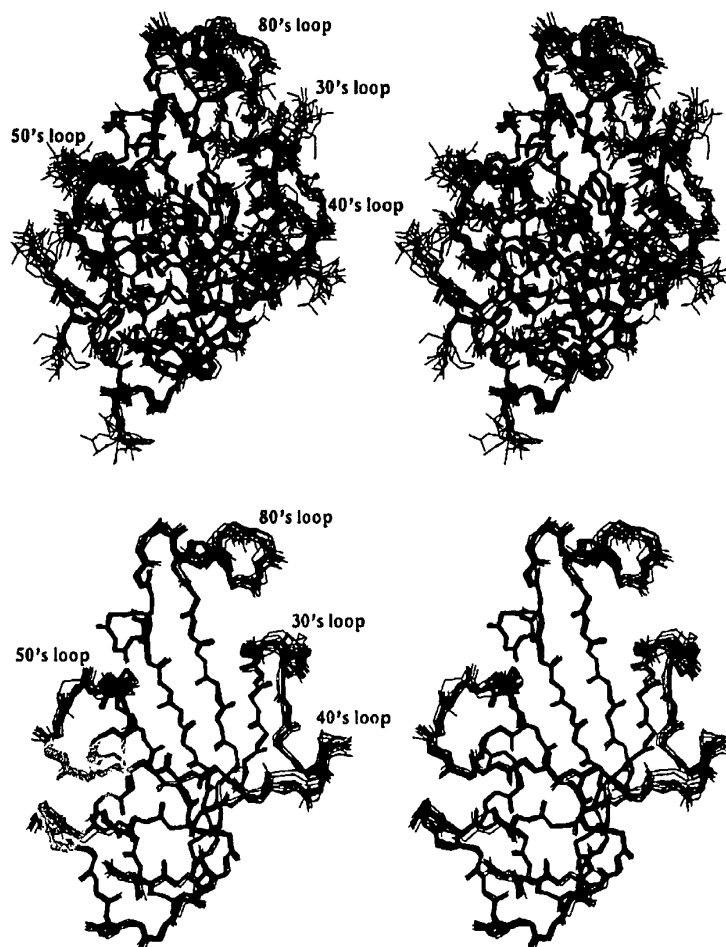


Fig. 1. Superposition of the coordinates of the 19 FKBP12 structures described in Table 1, with all main-chain and side-chain atoms shown, and with main-chain (C, N, O) atoms only. This comparison identifies regions of FKBP12 whose conformation is largely conserved (mostly localized in the framework β -sheet and α -helix secondary structure), and other regions that show variable conformation and mobility (mostly localized in loops). The correspondence between the 19 structures shown, all from different crystal forms, suggests that experimental artifacts such as crystal contacts are not responsible for the structural differences observed, and that the rigidity of the framework and the conformational flexibility of the loops may have functional relevance.

Rosenberg, Dunn & Dubendorff, 1990). Production of hrFKBP12 was initiated by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), to a final concentration of 1 mM. This induces a chromosomal copy of T7 RNA polymerase (behind the *lac* UV promoter) in the host JM109/DE3, which then initiates transcription of the protein gene (Studier & Moffat, 1986). The yield from a typical culture in a 10 l fermentor (Biostat ED. B. Braun) was about 250 g of wet cell paste. The cell paste was stored frozen at 203 K prior to protein purification. Full details of the hrFKBP12 expression will be presented elsewhere. (Chambers *et al.*, unpublished work)

Typically, about 500 g of frozen cell paste was thawed and slurried in four additional volumes of cold buffer [50 mM potassium phosphate, 1 mM EDTA, 1 mM β -mercaptoethanol and 0.1 mM phenylmethylsulfonylfluoride, pH (278 K) = 7.0]. The slurry was briefly homogenized by mechanical disruption in a Polytron blender, as above, and then the cells were fully lysed by three serial passages through a high-pressure laboratory homogenizer (Rannie, Mini-lab 8.30H) at 9000 psi. After each passage, the lysed cell suspension was collected on ice, and afterwards centrifuged at 12 000g for 50 min at 278 K. The clarified extract was passed through a 5 \times 30 cm column of Pharmacia Fast Flow DEAE Sepharose (equilibrated at 278 K in the cell-extraction buffer), and the column flow-through and wash were collected. This was concentrated by ultrafiltration and subjected to size-exclusion and hydrophobic interaction chromatography essentially as described for the bovine thymus protein. From the 500 g of cell paste, about 3.5 g of hrFKBP12 was obtained.

Protein characterization

Both bFKBP12 and hrFKBP12 were judged better than 99% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis and reversed phase high pressure liquid chromatography. Purity and enzymatic integrity were further confirmed by N-terminal sequence analysis, by analysis of (3 H)dihydro-FK506 binding activity (essentially as described by Park, Aldape, Futer, DeCenzo & Livingston, 1992), and by enzymatic analysis of the peptidyl-prolyl isomerase activity (essentially as described by Harrison & Stein, 1990). The specific peptidyl-prolyl isomerase and tacrolimus binding activities of both proteins were equal to or greater than those previously reported (Siekierka, Hung, Poe, Lin & Sigal, 1989; Harding, Galat, Uehling & Schreiber, 1989; Park *et al.*, 1992), and all of the peptidyl-prolyl *cis-trans* isomerase activity of each protein was inhibitable by FK506 and was unaffected by cyclosporin A. All buffer-exchange and protein concentration steps were carried out by ultrafiltration, in a stirred cell (5000 M, cut-off membrane) under N_2 pressure at 278 K. The protein was stored in 10 mM sodium cacodylate (pH 6.5), at 203 K until needed. All protein concentrations were

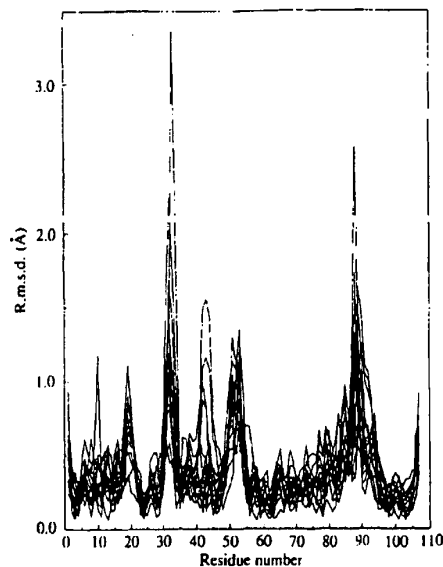


Fig. 2. Plot of the r.m.s. deviations in main-chain atom positions of the structures included in Table 1 after superposition onto the hrFKBP12-FK506 complex structure. The conformational flexibility in the loop regions is once again evident.

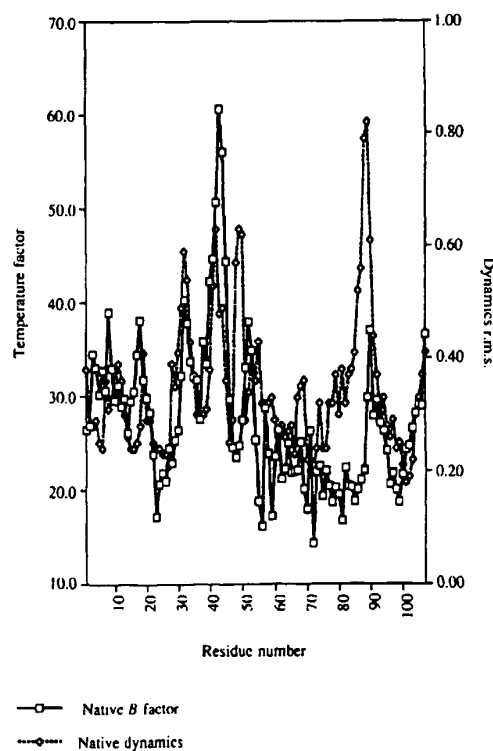


Fig. 3. Plot of the temperature factors (\AA^2) of the $C\alpha$ atoms of the structure of the unliganded bFKBP12 superimposed on the r.m.s. deviation motion of the $C\alpha$ atoms from the 100 ps dynamics runs on solvated unliganded bFKBP12.

determined using a molar absorption coefficient (A_{280}) of $9600\text{ M}^{-1}\text{ cm}^{-1}$

Preparation of FKBP12–drug complexes

Protein–drug complexes were prepared by addition of a drug stock solution (ligand at 20 mg ml^{-1} in dimethyl sulfoxide) to 1 mg ml^{-1} solutions of FKBP12 to achieve a protein:drug molar ratio of from 1:1.25 to 1:1.5. The protein complexes were then concentrated by ultrafiltration and washed with fresh 10 mM sodium cacodylate (pH 6.5) until the dimethyl sulfoxide concentration was less than 0.001% (v/v). The protein complexes were concentrated to $15\text{--}150\text{ mg ml}^{-1}$, depending on the observed solubility of each complex. Samples were finally centrifuged at 278 K for $20\text{--}30\text{ min}$ at about $30\,000g$ to remove microscopic debris, and stored at 253 K until crystallization trials began.

Structure determination of unliganded bFKBP12

Crystals of unliganded bFKBP12 were grown both in the laboratory and in a microgravity environment on board the United States Microgravity Laboratory-1 (USML-1) during mission STS-50 of the space shuttle orbiter Columbia, as described (DeLucas *et al.*, 1994). The crystallization conditions used were developed and optimized for earth-bound crystals. In both situations, tetragonal crystals of bFKBP12 ($P4_32_12$; $a = 34.38$, $c = 202.01\text{ Å}$) were grown at room temperature from 140 mg ml^{-1} protein solutions by vapor diffusion against $1.4\text{--}1.6\text{ M}$ sodium sulfate in 100 mM cacodylate buffer at pH 7.5; macroseeding was required for diffraction-quality crystals. On the whole, crystals of unliganded bFKBP12 diffract poorly when compared to crystals of FKBP12 complexes (Table 1). Crystal growth in microgravity significantly improved the signal-to-noise ratio and resolution of the diffraction data obtained,

however, as described previously (DeLucas *et al.*, 1994). All data were collected on an R-Axis II area detector (Rigaku/MSO, Woodland, TX). $10\,574$ reflections (3977 unique) were measured from a single crystal obtained from the microgravity experiment, to 2.3 Å resolution (68% of total available), with a symmetry R factor for the data of 4.9% on intensities. Initial phase information was obtained by Patterson correlation molecular replacement (Brünger, 1990a) using coordinates from an earlier structure determination of the FKBP12–FK506 complex (Van Duyne, Standaert, Karplus *et al.*, 1991); Protein Data Bank accession number 1FKF (Bernstein *et al.*, 1977). Alternating cycles of map interpretation using the program QUANTA (Version 3.3; Molecular Simulations Inc., 1993) and of simulated annealing with X-PLOR (Brünger, 1990b; Brünger, Krukowski & Erickson, 1990) were used to refine the model, with 67 water molecules built into difference electron density. Final refinement reduced the R factor to 15.8% at 2.3 Å resolution with reasonable geometry [deviation from ideality was 0.017 Å for bond lengths, 3.45° for bond angles and 1.90° for improper dihedrals; see Brünger (1990b) for definitions]. In contrast, an equivalent level of refinement (R factor = 15.9%) with similar geometry was only achievable at 3.1 Å resolution with unit-gravity data.

Structure of the bFKBP12–rapamycin complex

A 10 mg ml^{-1} solution of bFKBP12 in complex with rapamycin was crystallized by vapor diffusion against 50 mM ammonium sulfate and 200 mM potassium phosphate at pH 5.5, the space group is $P2_12_12_1$ with cell dimensions $a = 45.86$, $b = 49.46$, $c = 55.20\text{ Å}$. Crystals were similar to those reported earlier for the hrFKBP12–rapamycin complex (Van Duyne, Standaert, Schreiber *et al.*, 1991), even though bFKBP12 was used here instead. The crystal structure was solved

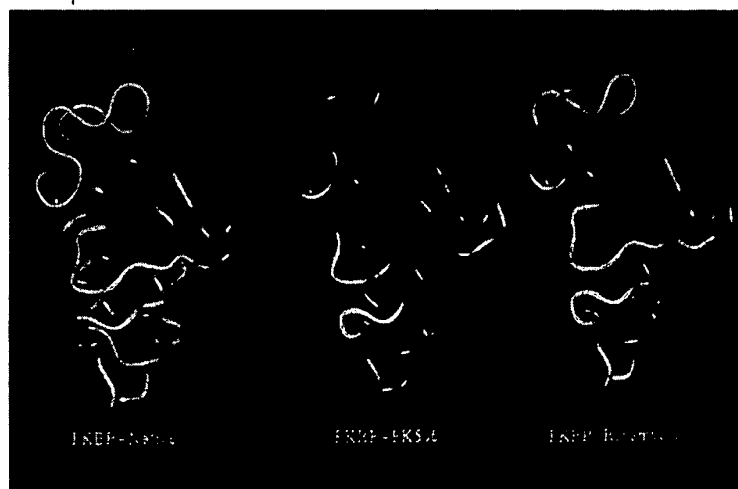


Fig. 4. Schematic comparison of the structures of unliganded bFKBP12, hrFKBP12 in complex with FK506, and bFKBP12 in complex with rapamycin. Structures are color coded by temperature factor; lowest in blue and highest in red. These data suggest that FK506 binding to FKBP12, which leads to a productive inhibition of calcineurin activity, may considerably restrict the overall mobility of FKBP12, in agreement with molecular-dynamics simulations.

by molecular replacement using coordinates from the hrFKBP12-FK506 complex structure described below. 11 083 unique reflections were observed to 1.77 Å resolution (90% of the total available). Refinement led to an *R* factor for the final model of 17.7%. Deviation from ideality was 0.016 Å for bond lengths, 2.94° for bond angles and 1.24° for improper dihedrals. The final atomic model consisted of 889 heavy protein atoms and 162 water molecules.

Structure of the hrFKBP12-FK506 complex

Crystals of the hrFKBP12-FK506 complex were obtained as described previously (Van Duyne, Standaert, Karplus *et al.*, 1991), except that the crystallization was carried out at pH 7.0 instead of pH 5.6. Crystals were grown by vapor diffusion from a 20 mg ml⁻¹ solution of hrFKBP12 in complex with FK506, against a reservoir of 1.7 M ammonium sulfate in 200 mM cacodylate at pH 7.0; the space group is *P*4₂2₁2 with cell dimensions *a* = 58.07, *c* = 55.65 Å. Data were collected and processed as above. 8743 unique reflections were observed to 1.76 Å resolution (90% of those available). Refinement was as described above, with starting coordinates taken from the earlier determination of the hrFKBP12-FK506 complex (Van Duyne, Standaert, Karplus *et al.*, 1991). Refinement led to a final *R* factor of 16.2% with good geometry (deviation from ideality was 0.014 Å for bond lengths, 2.87° for bond angles, and 1.15° for improper dihedrals). No significant differences were noted between the reported hrFKBP12-FK506 complex structure at pH 5.6 (Van Duyne, Standaert, Karplus *et al.*, 1991) and our structure at pH 7.0. The final atomic model consisted of 889 heavy protein atoms and 97 water molecules.

Structure comparisons

Structures of the unliganded FKBP12 and of the FKBP12 complexes with FK506 and with rapamycin were superimposed by a least-squares procedure within *X-PLOR* (Brünger, 1990b) that overlapped all main-chain atoms (C, N, O, Cα) of the protein. Calculated r.m.s. positional differences between the structures were 0.51 Å for the unliganded bFKBP12 versus the hrFKBP12-FK506 complex, 0.64 Å for bFKBP12 versus the bFKBP12-rapamycin complex, and 0.67 Å for the hrFKBP12-FK506 versus the bFKBP12-rapamycin complexes. Crystallization, structure determination and refinement parameters for these structures, and the 16 other inhibitor complex structures under discussion are summarized in Table 1.

Molecular-dynamics simulations

Molecular-dynamics simulations used the program *CHARMm* (Version 2.2) (Brooks *et al.*, 1983), as implemented within *QUANTA* (Version 3.3, Molecular Simulations Inc., 1993). Starting bFKBP12 coordinates were taken from this study. A subset of approximately ten crystallographically determined bound waters were included in the simulation, where the choice of these was based on thermal-factor data and visual examination of coordinates and maps. H atoms were added with *CHARMm*, and the resulting structures were energy minimized to a gradient of 0.04184 kJ Å⁻² (0.01 kcal Å⁻²). For these simulations, a distant-dependent dielectric ($\epsilon = r$) was used. Molecular dynamics were initiated by heating the structures from 0 K to 300 K during 5 ps, and then allowing the systems to equilibrate at 300 K for 25 ps. At that point, data accumulation was begun.

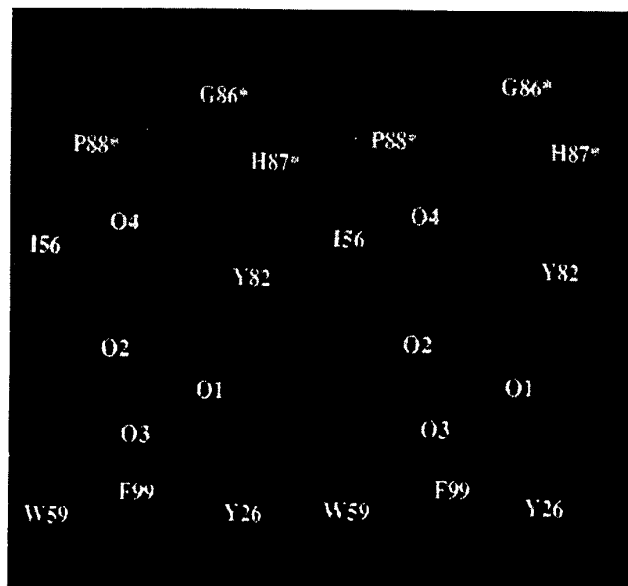


Fig. 5. Electron-density map of the ligand-binding cavity of unliganded bFKBP12, calculated with $2|F_o| - |F_c|$ 2.0 standard deviation units above background (blue), and with $|F_o| - |F_c|$ coefficients, contoured at 2.0 (yellow) and 3.0 (orange) standard deviation units above background. Superimposed on the density are the coordinates of the unliganded bFKBP12 (green) and of the hrFKBP12 complex with FK506 (violet). The excellent overall fit of these coordinates is indicative of how similar are the conformations adopted by the three FKBP12's in this region of the protein (for clarity, the rapamycin complex coordinates are not shown). Note that binding of FK506 (and of rapamycin) rotates the side chain of Trp59 when compared to the unliganded structure. The perturbation of Trp59 upon ligand binding may be responsible for the fluorescence changes previously reported on binding (Park *et al.*, 1992).

and continued for an additional 100 ps at 300 K with trajectories saved every 0.5 ps (for a total of 201 coordinate sets for each system). To test the stability of our model, native FKBP12 was subjected to an additional 200 ps of simulation, with trajectories saved every 0.5 ps. As a control, a fully solvated run was carried out on the native enzyme using a constant dielectric ($\epsilon = 1$). Approximately 200 water molecules were placed in the active site and a weak restraining force was used to prevent solvent drift. A non-bond cutoff of 12 Å was used for all simulations.

Results

Comparison of the structure of liganded and unliganded FKBP12's

In order to explore the possible mechanistic role of subtle conformational variability in FKBP12, we have solved the structures of unliganded bovine bFKBP12 (at 2.3 Å resolution, $R = 15.8\%$) and of bFKBP12 in complex with rapamycin (1.77 Å resolution, $R = 17.7\%$). Both are reported here for the first time. We have also re-determined independently the structure of the hrFKBP12 complex with FK506 (1.76 Å resolution, $R = 16.2\%$). Our analysis of protein conformation and mobility has been extended to include 16 other FKBP12 complex structures from our immunosuppressant drug design program (unpublished data), where these have been solved in different crystal forms with distinct packing arrangements and crystal contacts. These structures are summarized in Table 1, which lists space group, unit cell, data collection and refinement parameters. The ligands used were typically tight-binding linear diketopipercolate analogs, including the compound whose complex structure is described in the accompanying paper (Armistead *et al.*, 1995); none of these ligands is able to inhibit calcineurin in complex with FKBP12, however.

All 19 FKBP12 structures are superimposed in Fig. 1, which contrasts the close alignment found in the framework regions of the molecule with the mobility of the loops. Taken together with the r.m.s. deviation of main-chain atoms calculated for the 19 superimposed structures (Fig. 2), these data suggest that the observed variability in FKBP12 is intrinsic to the protein, and that FKBP12 is subject to significant localized changes in conformation and mobility as a consequence of ligand binding. Support for this view is derived from an analysis of molecular-dynamics simulations and from generalized order parameter data obtained by ^1H - ^{15}N NMR spectroscopy (Cheng *et al.*, 1993).

Molecular-dynamics simulations

Fig. 3 shows that there is general agreement between the r.m.s. deviation of C α -atom positions taken from the 300 ps molecular-dynamics simulation of solvated

unliganded FKBP12, and the scaled C α -atom temperature factors taken from the corresponding unliganded bFKBP12 structure. The molecular-dynamics simulation suggests that the '80's loop' (Fig. 1) delimited by Thr85 and Asn94 may move cooperatively, as a flap (see Fig. 3), with the variability for the φ - ψ angles of the residues at the ends of the loop suggesting a hinge. The overall mobility of the 80's loop can be monitored by the average distance from the C α atom of Trp59 at the base of the ligand-binding site to that of His87 at the tip of the 80's loop. In the unliganded FKBP12 simulation, the average distance is 18.2 Å with an r.m.s. deviation of ± 1.7 Å. For the FK506 complex, however, the average distance decreases to 16.7 Å and the r.m.s. deviation is only 0.8 Å. This restriction in flap mobility is apparently due to favorable van der Waals interactions between the pyran ring and its substituents on FK506 with residues His87 and Ile91 on the protein. Dynamics simulation of the FKBP12-rapamycin complex shows that the average distance and r.m.s. deviation between Trp59 and His87 is only slightly reduced when compared to the unliganded protein. Rapamycin has a different substitution pattern on the pyran ring and consequently makes fewer van der Waals interactions. This pattern of restricted mobility suggests that the structure of FKBP12 in complex with FK506 is significantly more ordered than either the rapamycin complex or the unliganded protein, in agreement with corresponding X-ray temperature-factor data (Fig. 4). NMR dynamics studies of free and FK506-bound FKBP12 (Cheng *et al.*, 1993) also show that the 80's loop becomes fixed on binding ligand.

Solvent structure in the active site

Few ordered water molecules are found in the generally hydrophobic environment of the binding cavity of unliganded FKBP12 (Fig. 5). Nonetheless, these solvent molecules appear to be important contributors to the binding of FKBP12 ligands and substrates. A comparative thermodynamic and structural study of wild-type and mutant (Tyr82Phe) hrFKBP12's in complex with FK506 (Connelly *et al.*, 1993) concluded that the expulsion of two of these water molecules made a favorable entropic contribution to the overall binding of FK506.

The C8 carbonyl O atom of FK506 (nomenclature as defined in the accompanying paper, Armistead *et al.*, 1995) is thought to resemble the *trans*-prolyl carbonyl of a peptide substrate; the C9 carbonyl O atom is thought to mimic the twisted amide transition state for the PPIase activity of FKBP12 (Schreiber, 1991; Rosen & Schreiber, 1992; Rosen, Standaert, Galat, Nakatsuka & Schreiber, 1990). In the structure of the complex with FKBP12, the C8 carbonyl O atom of FK506 forms a good hydrogen bond with Tyr82 OH (2.8 Å), while the C9 carbonyl O atom interacts with the edges of Phe36 (3.4 Å to CZ), Tyr26 (3.5 Å to CE1) and Phe99 (3.6 Å to CE1) (Van Duyne, Standaert, Karplus *et al.*,

1991; Van Duyne *et al.*, 1993; and this work). The electron density corresponding to water molecule O1 is centered 3.3 Å from Tyr82 OH, and 3.4 and 3.8 Å from CE2 and CZ of Phe36 and Phe99, respectively (Fig. 5). The temperature factor for O1 is high (55.8 Å^2), however, with diffuse density suggestive of multiple solvent sites, each of which may satisfy the interactions observed for FK506. Water molecule O2 occupies a small hydrophobic/aromatic cavity composed of Val55, Ile56, Phe46 and Trp59 with strong electron density, and is directly hydrogen bonded to the amide N atom of Ile56; O2 corresponds most closely to the C1 carbonyl O atom of FK506. Connected density to water molecules O3 and O4 is evident in this region when one contours at a lower level, however. This density partially overlaps the space occupied by bound FK506, which acts as a twisted amide peptide analog, and may be suggestive of a partially occupied dipeptide in the active site.

We also observe a distinct difference in the orientation of the side chain of Trp59 in the unliganded state when compared to that observed on binding of either FK506 (Fig. 5) or rapamycin (similar data not shown). The perturbation of Trp59 upon ligand binding may be responsible for the fluorescence changes previously reported (Park *et al.*, 1992).

Comparison of sequence and structural data

FKBP12 sequences across species as distant as mammals and fungi are strongly conserved, more so than the sequences of other FK506 binding proteins (FKBP's) within a species (Peattie *et al.*, 1992, and references therein). This is not surprising for those residues that are directly involved in ligand binding. However, many conserved residues are found on the distal side of the β -sheet framework of the molecule, well away from the binding cavity for immunosuppressant ligands and the prolyl isomerase active site. Some of these residues are

clearly involved in hydrophobic and salt-bridge interactions that may serve to stabilize the protein, but some have no obvious reason to explain their conservation, and may well be involved in as yet undiscovered interactions.

Synthesis of FK506 by its producing organism may confer a competitive advantage upon it, by inhibiting the FKBP12's of competing microbial species. As fungal metabolites (Kino *et al.*, 1987; Vezina *et al.*, 1975), however, neither FK506 nor rapamycin are likely themselves to be the endogenous ligand(s) that normally interact with the mammalian FKBP12's; nor is there currently any direct evidence for the existence of such. By looking at the pattern of contiguous conserved residues in the structure of FKBP12, however, one can discern what might be the 'footprint' of such an endogenous ligand (Fig. 6), in the form of a continuous slot extending beyond the FK506-binding site. The slot is largely hidden by residues of the 80's loop, but these have been shown to behave as a mobile flap in the molecular-dynamics simulations described above. As with the aspartyl proteases (Davies, 1990; Wlodawer & Erickson, 1993) and various lipases (Cambillau & van Tilbeurgh, 1993), conserved structural elements protected by a flap hint at functionality (Sali *et al.*, 1992), and one can speculate on the possible displacement of the flap as a component of the binding of the unknown endogenous ligand, or of calcineurin. Site-directed mutagenesis of the conserved residues of the footprint region may clarify their role in the function of FKBP12.

Discussion

The differences in FKBP12 structure that we have described suggest that the conformation of the protein may be important in establishing the calcineurin inhibitory potential of a given ligand complex, and that immunosuppressive ligands like FK506 may express

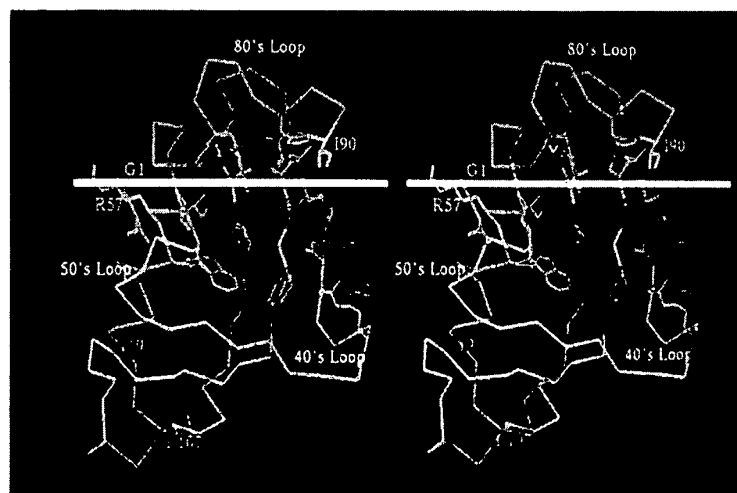


Fig. 6. Ca trace of the structure of unliganded bFKBP12 shown in stereo. Conserved residues associated with FK506 binding are shown in blue; conserved residues contiguous to the latter are in yellow; contiguous backbone atoms are in orange. The combined pattern of conserved residues is suggestive of a 'footprint' of interaction for the putative endogenous FMBP12 ligand that FK506 may mimic. The footprint of conserved residues on the surface of FKBP12 forms a slot that extends away from the FK506 binding cavity to the surface of the protein. The slot is protected by residues Thr85 to Asp94, which molecular-dynamics simulations suggest may be mobile as a unit, acting as a 'flap', such as is found, for example, in HIV-1 protease and other members of the aspartyl protease family (see text).

their differential effects in part by modulating the protein conformation. This point is illustrated by the breakdown of the effector-domain model in explaining the remarkable behavior of L-658,818 (a close 18-hydroxyl-21-ethyl analog of FK506) (Rotonda *et al.*, 1993; Becker *et al.*, 1993; Parson *et al.*, 1993). In complex with hrFKBP12, L-658,818 acts as an antagonist of calcineurin inhibition, much like rapamycin; in complex with yeast FKBP12, however, L-658,818 becomes a more potent inhibitor of calcineurin phosphatase activity than FK506 itself (Rotonda *et al.*, 1993; Parson *et al.*, 1993). The Merck group has compared the structures of FK506* in complex with human and yeast FKBP12's, respectively, and has found, in addition to the obvious side-chain substituents, main-chain r.m.s. differences corresponding to those found in the regions of mobility identified in this study (Figs. 1–4). Least affected by its binding to the human and yeast FKBP12's was the conformation of FK506 itself, with an r.m.s. deviation between structures of only 0.35 Å (Rotonda *et al.*, 1993), a figure that is roughly comparable to the expected error of the method (Rotonda *et al.*, 1993; Becker *et al.*, 1993).

The structures of mutant FKBP12's in complex with FK506 (Itoh *et al.*, 1995; Lepre *et al.*, unpublished work), along with the limited functional data available for FK506 analogs (Tocci *et al.*, 1989; Sigal & Dumont, 1992; Armistead & Harding, 1993; Orkun *et al.*, 1993; Dumont *et al.*, 1992; Parson *et al.*, 1993; Kawai *et al.*, 1993; Goulet *et al.*, 1994), all support the existence and importance of a composite surface of ligand and protein structural elements whose integrity is critical for calcineurin inhibition (Aldape *et al.*, 1992). Within this interaction region (Fig. 7), we have identified several prominent depressions on the surface of the FKBP12–FK506 complex that are either distorted or blocked outright in antagonist complexes. Significantly, the constituent amino acids that form a part of these composite depressions are those whose mutation most severely affects calcineurin inhibition by the corresponding FKBP12–FK506 complex, and whose location can also be mapped to the regions of FKBP12 conformational variability that we have identified (Figs. 1–4) in this study.

Concluding remarks

The foundations of the effector-domain model depend on a correlation between the agonist and antagonist behavior of FK506 and rapamycin in promoting calcineurin inhibition (Schreiber, 1991; Rosen & Schreiber, 1992; Schreiber & Crabtree, 1992; Liu, 1993a,b; Schreiber

et al., 1993), and the differences in structure between the two ligands in their respective FKBP12 complexes (Van Duyne, Standaert, Karplus *et al.*, 1991; Van Duyne, Standaert, Schreiber *et al.*, 1991; Van Duyne *et al.*, 1993). If one is to generalize the model beyond those defining examples and specifically, if one is to attribute a special role to the particular FK506 effector-domain conformation that is seen in its FKBP12 complex, then it becomes necessary to assume that the protein remains 'nearly identical' (Schreiber *et al.*, 1993) in the various possible ligand bound and unbound states, and hence functionally irrelevant to any of them. These assumptions have been particularly attractive with respect to structure-based drug design in this area, since they justify simple mimicry of the FK506 effector-domain conformation as a target strategy (see, e.g., the accompanying paper, Armistead *et al.*, 1995, and references therein).

Our results suggest that the interactions involved in calcineurin inhibition by FKBP12–ligand complexes are more complicated than previously recognized, and

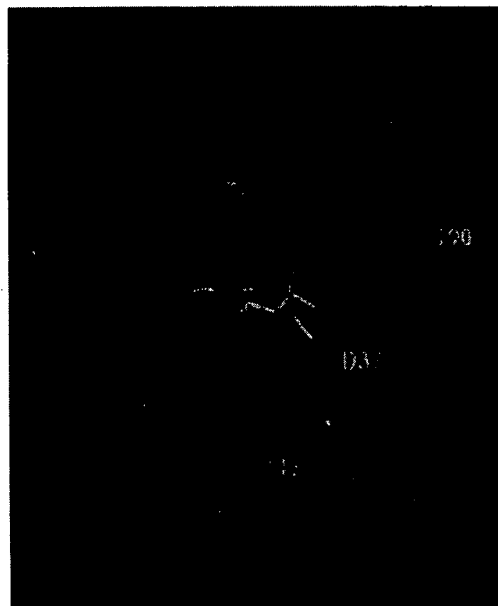


Fig. 7. Solvent-accessible surface representation of the FKBP12–FK506 complex structure. The surface is color coded with respect to its curvature, from concave (in red) to convex (in blue), as calculated by the program GRASP (Nicholls & Honig, 1992). Side chains for those residues known to be involved in calcineurin inhibition by site-directed mutagenesis studies (Aldape *et al.*, 1992; Yang, Rosen & Schreiber, 1993) are also displayed. A number of suggestive depressions are found in the functionally important region of the complex surrounding the bound ligand, including the 'catcher's mitt' (see text) formed by FK506 and protein residues Asp37, His87 and Ile90. A second depression involves residues Arg42 and Phe46. Ligand-induced conformational changes, or mutations (Aldape *et al.*, 1992; Itoh *et al.*, 1995) in this mobile region of the protein could alter the conformation of these surface depressions (or could eliminate them altogether), thereby preventing the binding and inhibition of calcineurin.

* The conformation of L-685,818 strongly resembles that of FK506 in its complex with human recombinant FKBP12 (r.m.s. Δ for all atoms = 0.35 Å, Becker *et al.*, 1993). As such, it was reasonably assumed that L-685,818 would adopt the same conformation found for FK506 in the yeast complex (Rotonda *et al.*, 1993). The structure of yeast FKBP12 in complex with L-685,818 has not been reported, however.

that local conformational flexibility may be an intrinsic characteristic of FKBP12 that has functional implications. In this context, the observed antagonism of the synthetic effector-less ligand 506BD (Bierer *et al.*, 1990; Somers *et al.*, 1991), though critical in gaining acceptance for the model, fails to support the proposed sufficiency of the effector-domain in promoting agonist calcineurin inhibitory activity. In addition, the structural and biochemical consequences on calcineurin inhibition of FK506 analogs like L-688,518 in complex with FKBP12 (Rotonda *et al.*, 1993; Becker *et al.*, 1993; Parson *et al.*, 1993), and of FK506 itself in complex with site-directed mutants of FKBP12, are consistent with a critical role for the FKBP12 protein, but are difficult to reconcile with the effector-domain hypothesis as it stands.

Success in the design of novel immunosuppressants that operate through the calcineurin pathway may have to take specific account of the participation of the FKBP12 protein in the interaction with calcineurin, and may ultimately require that we reconsider the design process as an enzyme inhibitor problem in the context of a solved structure of the FKBP12-FK506 complex bound to calcineurin itself.

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